

## Local Expression of the Serum Amyloid A and Formyl Peptide Receptor–Like 1 Genes in Synovial Tissue Is Associated With Matrix Metalloproteinase Production in Patients With Inflammatory Arthritis

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**Objective.** To evaluate the regulation of acute-phase serum amyloid A (A-SAA) production in inflamed synovial tissue, and to elucidate a possible pathophysiologic role in the induction of matrix metalloproteinase (MMP) release by fibroblast-like synoviocytes (FLS).

**Methods.** Synovial tissue samples were obtained by arthroscopic biopsy from the knee joints of patients with inflammatory arthritis. Primary cultures of FLS from patients with rheumatoid arthritis (RA), psoriatic arthritis, sarcoid arthritis, and undifferentiated arthritis were established. Total RNA was extracted from FLS and analyzed by reverse transcription–polymerase chain reaction (PCR) using specific primers for A-SAA and formyl peptide receptor–like 1 (FPRL1), an A-SAA receptor. Southern blot analysis confirmed the PCR products generated. Immunohistochemical analysis demonstrated the expression of A-SAA protein production by several synovial cell populations, and immunofluorescence analysis confirmed A-SAA colocalization with the macrophage marker CD68. Primary FLS cultures stimulated with recombinant human A-SAA resulted in dose-dependent MMP-1 and MMP-3 production, as measured by an enzyme-linked immunosorbent assay.

**Results.** A-SAA messenger RNA (mRNA) and FPRL1 mRNA were present in FLS, macrophages, and

endothelial cells isolated from the synovial tissue of patients with RA and other categories of inflammatory arthritis. A-SAA expression was regulated by proinflammatory cytokines and occurred in association with FPRL1 expression in FLS and endothelial cells, which is consistent with a biologic role at the sites of inflammation. Recombinant human A-SAA induced both MMP-1 and MMP-3 secretion by FLS. The mean fold increases in A-SAA–induced MMP-1 and MMP-3 production were 2.6 and 10.6, respectively, compared with 7.6-fold and 41.9-fold increases in interleukin-1 $\beta$ –induced MMP-1 and MMP-3 production.

**Conclusion.** The up-regulation of the A-SAA and FPRL1 genes in inflamed synovial tissue suggests an important role in the pathophysiology of inflammatory arthritis. A-SAA induces the production of MMPs. Therapeutic targeting of A-SAA, or FPRL1, may modulate pathophysiologic pathways that are associated with matrix degradation in patients with RA and other forms of progressive inflammatory arthritis.

Rheumatoid arthritis (RA) is characterized by varying levels of inflammation in synovial tissue (for review, see ref. 1). The varying levels of inflammation in RA are reflected by alterations in the acute-phase response, a nonspecific phenomenon induced by inflammation, infection, and tissue damage (2). The acute-phase response is characterized by altered plasma concentrations of several proteins, some of which decrease, including apolipoprotein A-I (Apo A-I), and some of which increase, including serum amyloid A (SAA). Acute-phase proteins are produced predominantly by the liver and have a critical role in host defense mechanisms (3). SAA is a family of small homologous apolipoproteins that range from 12 kd to 14 kd. They are

Supported by the Health Research Board of Ireland.

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Submitted for publication March 4, 2003; accepted in revised form February 25, 2004.

encoded by different genes with high allelic variation and high degrees of homology between species. The SAA proteins in mammals are well conserved throughout evolution.

There are 4 SAA genes in the human genome (4). SAA1 and SAA2 are specific to the acute-phase response and are collectively termed acute-phase SAA (A-SAA) (5). They share >95% nucleotide identity in their exon, intron, and promoter regions (6). SAA3 is known to be a pseudogene (a DNA sequence that, despite being largely homologous to a transcribed sequence elsewhere in the genome, is not transcribed) in humans; SAA4 is constitutively expressed in humans and is not hyperinducible. SAA1 and SAA2 are coordinately regulated and are arranged "head-to-head" in a gene cluster that also contains SAA3 and SAA4 on chromosome 11p15.1 (3). Following secretion by hepatocytes, A-SAA associates rapidly with the high-density lipoprotein fraction 3, from which it displaces Apo A-I (2,7,8). Serum levels of A-SAA reach concentrations of up to 1 mg/ml. Extrahepatic production of A-SAA has also been observed in several normal human tissues (9–11). Abundant production of A-SAA by fibroblast-like synoviocytes (FLS) isolated from synovial tissue samples obtained from patients with RA and other inflammatory arthropathies has been recently described (12,13).

A-SAA can bind to formyl peptide receptor-like 1 (FPRL1), a 7-transmembrane G protein-coupled receptor (a receptor that spans the membrane 7 times) that was first identified on human mononuclear phagocytes (14). FPRL1 is also expressed on nonphagocytic cells, including hepatocytes (15), and is highly inducible by a number of cytokines, including interleukin-13 (IL-13) and interferon- $\gamma$  (IFN $\gamma$ ), in epithelial cells (16). In addition to A-SAA, FPRL1 also interacts with a diverse array of other exogenous and endogenous ligands that are involved in inflammatory and host defense mechanisms. The lipid metabolite lipoxin A<sub>4</sub> also binds to FPRL1, which antagonizes the inflammatory response (14,17). Thus, A-SAA-induced monocyte and neutrophil migration to inflamed tissues is mediated through FPRL1 (14). A-SAA is also known to induce matrix metalloproteinase (MMP) production by synovial tissue FLS (18–20), but it is not known whether this is mediated through FPRL1 binding. At nanomolar concentrations, lipoxin A<sub>4</sub> inhibited IL-1 $\beta$ -induced MMP-3 production (21).

The aims of this study were to further characterize the cells that produce A-SAA in inflamed human synovial tissue, to determine whether FPRL1 is also expressed on synovial cell populations, and to investigate

the role of A-SAA in MMP-1 and MMP-3 production in RA and related disorders. We demonstrated A-SAA messenger RNA (mRNA) expression in FLS and synovial endothelial cells and A-SAA protein production by synovial tissue macrophages, in several categories of inflammatory arthritis. A-SAA mRNA and FPRL1 mRNA were coordinately up-regulated by proinflammatory cytokines. Finally, recombinant human A-SAA induced both MMP-1 and MMP-3 production by FLS. These findings provide further insights into the relationships between synovial tissue inflammation and the mechanisms that result in cartilage and bone matrix degradation.

## PATIENTS AND METHODS

**Clinical and demographic details.** Patients with inflammatory polyarthritis were recruited to the study from the rheumatology outpatient clinics at St. Vincent's University Hospital. A diagnosis of RA and psoriatic arthritis (PsA) was established according to widely accepted criteria (22,23). Clinical details were recorded according to a predesigned protocol. Patients were required to have clinical involvement of at least one knee joint. Synovial tissue samples were obtained by arthroscopic biopsy from the knee joints of 14 patients with inflammatory arthritis. Eight patients had seropositive RA, 3 had PsA, 1 had sarcoid arthritis, and 2 had an undifferentiated seronegative arthritis. The mean disease duration was 21.8 months (range 2–72 months). The mean age of the patients was 45.5 years (range 21–63 years); 10 patients were male. Most patients were receiving nonsteroidal antiinflammatory drugs, but none had ever received a disease-modifying antirheumatic drug (DMARD) or corticosteroid therapy. None had ever received an intraarticular corticosteroid injection. Synovial tissue samples were also obtained from an additional 3 patients with seropositive RA who were undergoing knee joint arthroplasty. Histologically normal synovium was obtained from the knee joint of a patient undergoing lower limb amputation. All patients gave written informed consent before undergoing synovial biopsy. The study was approved by the institutional ethics committee.

**Synovial biopsies.** Synovial biopsy of one knee joint was performed under sterile conditions using a Storz 2.7-mm needle arthroscope (Karl Storz, GmbH & Co., Tuttlingen, Germany) (24). Samples were selected from areas that were as close as possible to the cartilage-pannus junction without causing damage to the adjacent cartilage. Synovial tissue samples were immediately snap-frozen in embedding medium at optimal cutting temperature (Tissue-Tek; Miles, Elkhart, IN) by immersion in liquid nitrogen and were stored until used.

**Isolation and culture of synovial tissue FLS and endothelial cells.** FLS cells were obtained by enzymatic digestion of synovial membrane with 1 mg/ml type 1 collagenase (Worthington Biochemical, Freehold, NJ) in RPMI (Gibco BRL, Paisley, UK) for 4 hours at 37°C in 5% CO<sub>2</sub>. Dissociated cells were plated in RPMI supplemented with 10% fetal calf serum (Gibco BRL), 10 ml of 1 mmol/liter HEPES (Gibco BRL), penicillin (100 units/ml), streptomycin (100 units/ml),

and fungizone (0.25  $\mu\text{g/ml}$ ). The cells were grown to confluency ( $\sim 10$  days) at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  atmosphere then harvested with trypsin and passaged. FLS were found to be morphologically homogeneous fibroblast-like cells and were used between the third and seventh passages. To confirm that FLS cultures were not contaminated by monocytes, staining for the monocyte marker CD14 was carried out. All cells were placed in serum-free medium 24 hours prior to stimulation. Synovial membrane endothelial cells (SMECs) were isolated from synovial tissue specimens using methods previously described (25) and were characterized by flow cytometry using an antibody to CD31 (Research Diagnostics, Pleasant Hill, NJ), an endothelial cell marker.

**Treatment of primary FLS cultures with recombinant human A-SAA.** Primary cultured FLS were plated to a cell count between  $0.1 \times 10^6$  and  $0.5 \times 10^6$ . Having reached 80–90% confluency, the FLS were placed in serum-free media overnight. The FLS were treated with varying concentrations of recombinant human apolipoprotein SAA (1–150  $\mu\text{g/ml}$ ) or IL-1 $\beta$  at a concentration of 10 ng/ml.

**Synovial explant culture.** Synovial tissue specimens ( $\sim 20 \text{ mm}^3$ ) from patients with RA were minced aseptically and placed into 6-well culture plates (growth area  $9.6 \text{ cm}^2$ ) (BD Biosciences, San Jose, CA), in serum-free RPMI supplemented with 10 ml of 1 mmol/liter HEPES (Gibco BRL), penicillin (100 units/ml), streptomycin (100 units/ml), and fungizone (0.25  $\mu\text{g/ml}$ ). The samples were incubated in serum-free media for 24 hours at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  atmosphere prior to treatment.

**Preparation of recombinant SAA and cytokines.** The lyophilized recombinant human apolipoprotein acute-phase SAA (A-SAA) (PeproTech, London, UK) corresponding to A-SAA was reconstituted according to the manufacturer's instructions. This was endotoxin-tested, and levels were determined to be  $<0.1 \text{ ng/ml}$  (1 endotoxin unit/ $\mu\text{g}$ ). IL-1 $\beta$ , 5  $\mu\text{g}$  (catalog no. 407615; Calbiochem, Nottingham, UK), was received as a lyophilized solid containing 50  $\mu\text{g}$  of human serum albumin/ $\mu\text{g}$  IL-1 $\beta$ . IL-1 $\beta$  was resuspended in 1 ml of phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). IL-6, 10  $\mu\text{g}$  (catalog no. 1131567; Roche Diagnostics, Lewes, UK), was diluted with PBS containing BSA (1 mg/ml). The final concentration used in each experiment was 10 ng/ml. Tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), 10  $\mu\text{g}$  (catalog no. 654205; Calbiochem), was received as a lyophilized solid containing 50  $\mu\text{g}$  BSA/ $\mu\text{g}$  TNF $\alpha$ . TNF $\alpha$  was reconstituted to a concentration of  $>10 \mu\text{g/ml}$  with sterile PBS containing 0.1% BSA. All of the cytokines, once hydrated and aliquoted, were stored at  $-70^\circ\text{C}$ .

**RNA extraction from primary cultured FLS and synovial biopsy specimens.** Total RNA was extracted from FLS using the RNeasy total RNA isolation protocol (Qiagen, Crawley, UK). The RNA STAT-60 protocol of Tel-Test "B", Inc. (catalog no. CS-111; Tel-Test, Friendswood, TX) was used to extract total RNA from synovial biopsy specimens. Ethidium bromide staining of 2% agarose gels was used to determine the integrity of the total RNA. Ethidium bromide-stained gels were visualized using the Eagle Eye II still video system (Stratagene, Amsterdam, The Netherlands).

**Reverse transcription-polymerase chain reaction (RT-PCR).** Total RNA, isolated from freshly obtained synovial biopsy specimens or cultured primary FLS, was converted by

reverse transcription into complementary DNA (cDNA) (see ref. 13 for a detailed description of the RT-PCR technique). Specific FRPL1 were designed to generate a 1.1-kb product: sense primer 5'-CACCAGGTGCTGCTGGCAAG-3' and antisense primer 5'-AATATCCCTGACCCATCCTCA-3'. The cycle number for each of the 3 primer pairs used was as follows: for GAPDH, 35 cycles; for A-SAA, 35 cycles; for FPRL1, 35 cycles. This 35-cycle regimen for the GAPDH primers ensured that the PCR had not reached the plateau phase of amplification. All of the primer pairs that were used span an intron; thus, the PCR generated an unequivocally RNA-derived band.

**Southern blot analysis.** PCR products generated were run on a 2% agarose gel and transferred onto a nylon membrane (BioRad, Richmond, CA) using standard procedures. Human A-SAA, FPRL1 (a gift from Dr. C. Godson, Mater Hospital, Dublin), and GAPDH (26) cDNA probes were radiolabeled to a high specific activity using  $\alpha$ - $^{32}\text{P}$ -labeled dCTP and a random labeling system (Promega, Madison, WI). All membranes were probed under high stringency conditions. Blots were exposed to film at  $-80^\circ\text{C}$  using intensifying screens.

**Immunohistochemical analysis.** Synovial tissue was placed in the cryopreservative at optimal cutting temperature and immediately frozen in liquid nitrogen. Synovial tissue sections were stained according to the methods described by O'Hara et al (13). The primary antibody (1:1,200–1:1,600 dilution) for A-SAA was a polyclonal rabbit anti-human antibody and was incubated for 1 hour at room temperature. Biotinylated secondary antibodies (anti-rabbit) (Vectastain; Vector, Peterborough, UK) were prepared and added to the relevant sections and incubated for 30 minutes. The secondary antibody was washed off, and the slides were incubated with avidin-biotin complex (Vectastain; Vector) solution for 30 minutes and incubated for 6 minutes with 3,3'-diaminobenzidine and counterstained in hematoxylin stain for 1 minute. The sections were mounted using DPX (BDH Laboratory Supplies, Poole, UK). For negative control, isotype-matched nonimmune IgG was included.

**Immunofluorescence microscopy.** The primary monoclonal antibody for A-SAA (mouse anti-human; Biomedica, Foster City, CA) (1:100 dilution) was incubated for 1 hour at room temperature. This was followed by the addition of biotinylated anti-mouse secondary antibody (Vectastain; Vector) to the relevant sections and incubation for 30 minutes. The sections that were to be dual stained were incubated for 1 hour with a monoclonal CD68 fluorescein isothiocyanate-conjugated (Santa Cruz Biotechnology, Santa Cruz, CA) antibody. A 1:4,000 dilution of C3-streptavidin (Jackson ImmunoResearch, West Grove, PA) was prepared and applied to the dual-stained sections for 30 minutes. The sections were then mounted using Dako fluorescent mounting medium containing 15 mM  $\text{NaN}_3$  (Dako, Carpinteria, CA). Isotype-matched nonimmune IgG was included as a control for each of the primary antibodies.

**Measurement of MMP-1 and MMP-3 by enzyme-linked immunosorbent assay.** MMP-1 and MMP-3 protein in supernatants derived from FLS of patients with primary RA were harvested and levels were measured by a sandwich enzyme immunoassay kit (Amersham Pharmacia Biotech, Little Chalfont, UK) according to the manufacturer's instructions. The absorbance was measured at 450 nm in a microtiter plate

spectrophotometer (Dynatech MR4000; Dynatech, Alexandria, VA). Color intensity is directly proportional to the amount of MMP in the sample, and the concentration of MMP in a sample is determined by interpolation from a standard curve. The detection limits of MMP-1 and MMP-3 assays were determined to be 2.35 ng/ml and 1.7 ng/ml, respectively. In control experiments, supernatants were harvested for analysis at 24 hours.

**Statistical analysis.** Data are expressed as the mean  $\pm$  SEM. Comparisons made between treated and untreated synovial tissue samples were made using Wilcoxon's signed rank test for paired values.

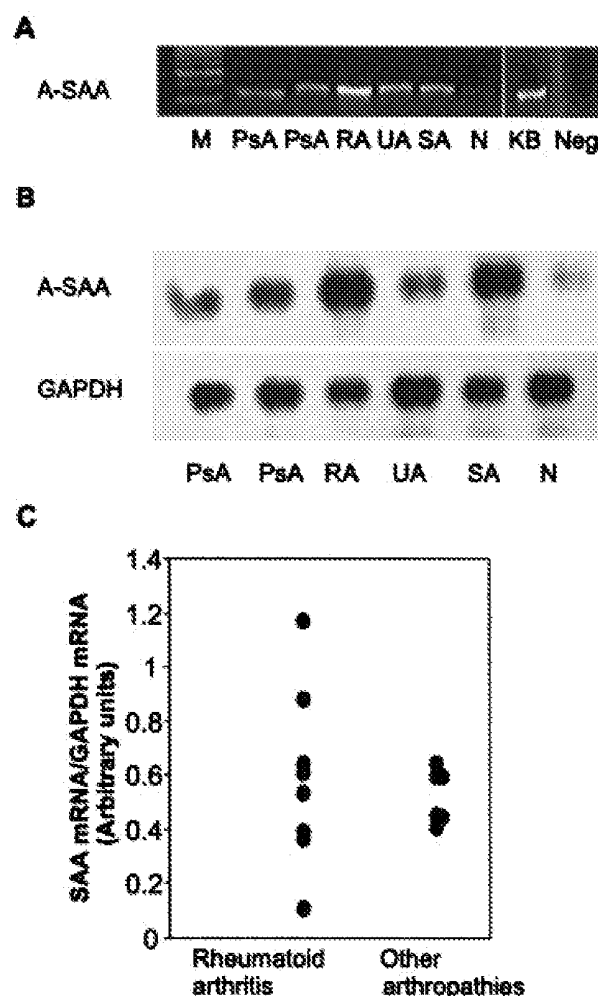
## RESULTS

**A-SAA gene expression in inflamed synovial tissue.** A-SAA mRNA expression was detected by RT-PCR in undigested synovial tissue samples from all of the patients with inflammatory arthritis. Synovial tissue A-SAA mRNA expression in 5 samples from patients with early arthritis is shown in Figure 1A. The level of A-SAA mRNA expressed by the normal synovial tissue sample was relatively low. The specificity of the cDNA generated was confirmed by Southern blot analysis using a probe that was specific for A-SAA (Figure 1B). The level of A-SAA mRNA expression varied among RA samples (Figure 1C).

In order to identify the cell populations that express A-SAA mRNA, RT-PCR was performed on RNA extracted from human primary synovial cell cultures generated from 6 arthroscopic biopsy samples. A-SAA mRNA expression was detected in all of the primary FLS that were examined ( $n = 13$ ). Figure 2A illustrates the A-SAA mRNA expression in 2 representative FLS cultures from patients with RA. RT-PCR was also performed on total RNA extracted from endothelial cells isolated from synovial membrane, which clearly demonstrated that A-SAA mRNA is present in SMECs (25) (Figure 2B). GAPDH, a housekeeping gene, was constitutively expressed in all the samples that were examined.

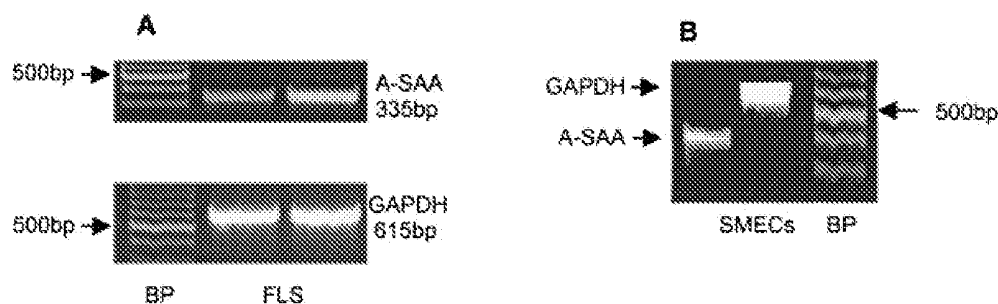
**A-SAA protein production by inflamed human synovial tissue.** A-SAA production by synovial tissue cell populations was further examined using immunohistochemical and immunofluorescence techniques. A-SAA protein was detected in all of the RA and PsA tissue samples examined by immunohistochemistry ( $n = 8$ ) (Figures 3A and B). Positive A-SAA staining was observed in vascular endothelial cells and in inflammatory cells, accumulating in both the synovial lining and sublining layers of the tissue (Figure 3).

To identify the inflammatory cells expressing immunoreactive A-SAA, synovial biopsy sections were



**Figure 1.** Semiquantitative analysis of acute-phase serum amyloid A (A-SAA) mRNA expression in human synovial tissue. Total RNA was analyzed by reverse transcription-polymerase chain reaction (RT-PCR) using primers specific for A-SAA and GAPDH. **A**, Representative example of A-SAA mRNA production in synovial tissue obtained from individual patients with various categories of arthritis (psoriatic arthritis [PsA], rheumatoid arthritis [RA], undifferentiated arthritis [UA], sarcoid arthritis [SA]), and from normal tissue (N); KB oral epidermal cell mRNA (KB) served as a positive control to ensure the efficacy of the A-SAA primers. For the negative control (Neg), no cDNA was added to the PCR reaction. M = molecular markers. **B**, Examples of RT-PCR products generated from synovial tissue from individual patients were confirmed by Southern blot analysis using cDNA probes for human A-SAA and GAPDH. **C**, Range of A-SAA mRNA levels in 8 patients with rheumatoid arthritis and 6 patients with other arthropathies, comparing A-SAA mRNA levels with those of GAPDH mRNA.

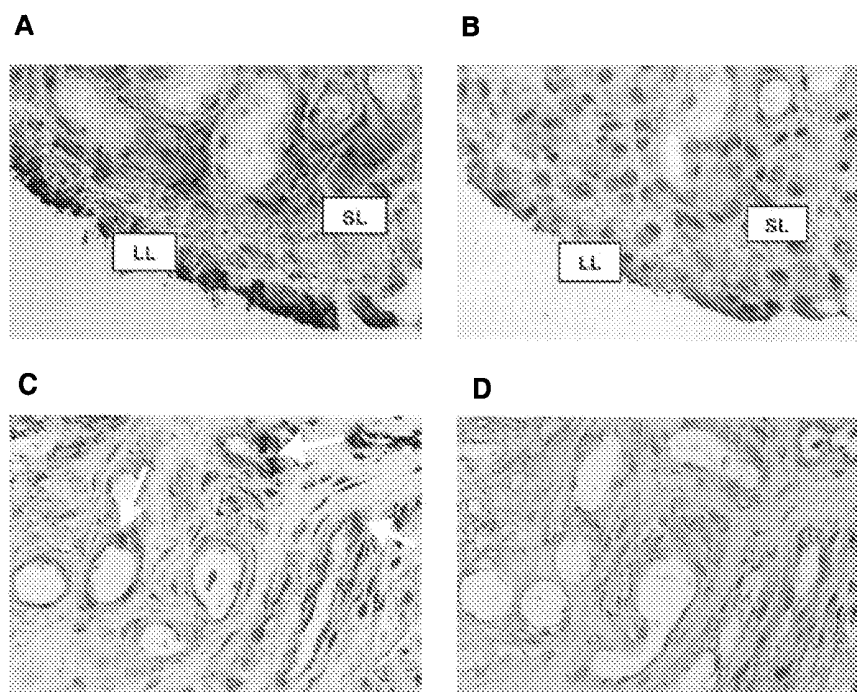
immunostained using a double-antibody staining technique. Colocalization of A-SAA staining with CD68+ cells was detected by dual immunofluorescence in all of



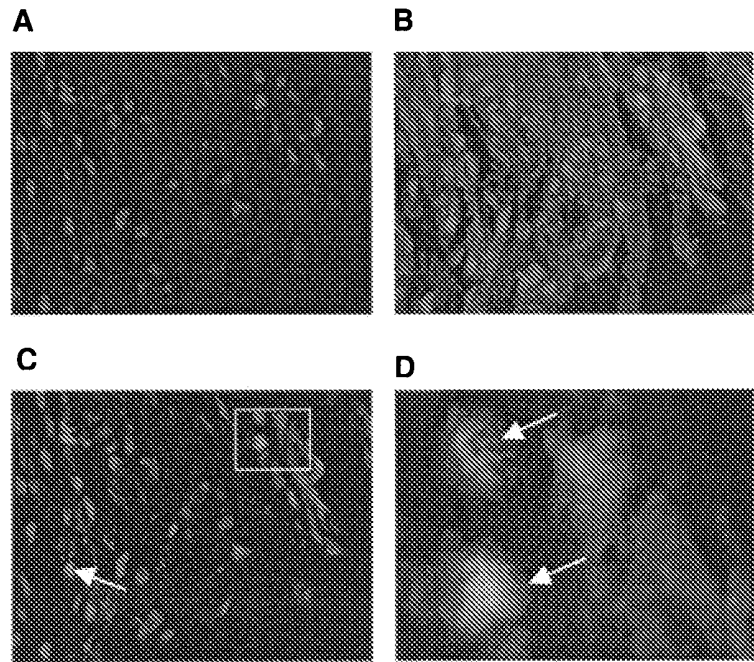
**Figure 2.** A-SAA mRNA expression in isolated primary rheumatoid arthritis fibroblast-like synoviocytes (FLS). **A**, Representative examples illustrating A-SAA mRNA expression detected by RT-PCR. **B**, Isolated primary synovial membrane endothelial cells (SMECs). Basepair (BP) markers are also shown. See Figure 1 for other definitions.

the tissues examined ( $n = 6$ ), indicating that synovial tissue macrophages are an additional source of A-SAA production (Figure 4). The patterns of immunohistochemical and immunofluorescent staining did not appear to differ between the clinical categories of arthritis (data not shown).

**FPRL1 gene expression in inflamed synovial tissue.** In order to elucidate the biologic role of A-SAA at peripheral sites of inflammation, expression of A-SAA receptor FPRL1 mRNA in inflamed human synovial tissue was investigated. FPRL1 mRNA expression was detected by RT-PCR in undigested synovial



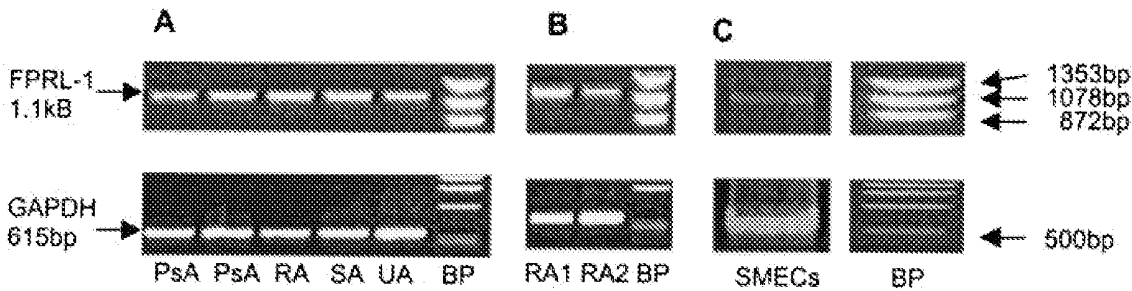
**Figure 3.** Immunohistochemical staining for acute-phase serum amyloid A (A-SAA) protein expression in inflamed synovial tissue. Psoriatic arthritis synovial tissue sections were stained with an antibody to A-SAA (**A** and **C**) or isotype-matched IgG (**B** and **D**). A blackish-brown stain indicates positive cells. A-SAA-positive cells are seen in the lining layer (LL), vascular endothelial cells, and perivascular regions. **Arrows** indicate A-SAA-positive vascular endothelium. SL = sublining layer. (Original magnification  $\times 100$ .)



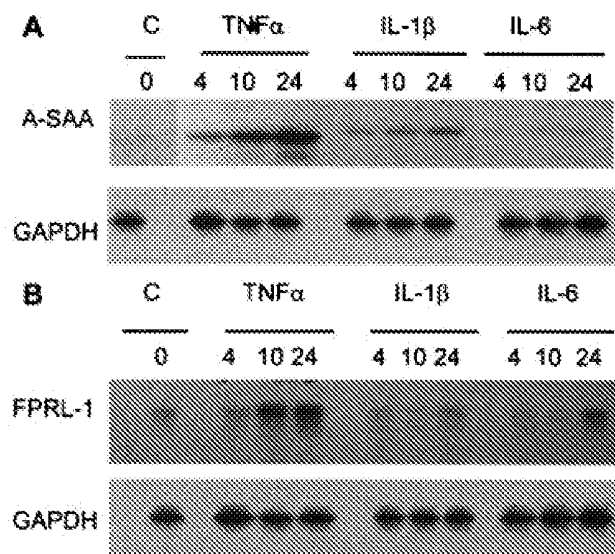
**Figure 4.** Colocalization of acute-phase serum amyloid A (A-SAA) protein expression with CD68-positive macrophage cells. Rheumatoid synovial tissue sections were dual stained using antibodies that were specific for SAA protein and anti-CD68, which is a marker for mature macrophage cells. **A**, A-SAA-positive staining is indicated by intense red staining. **B**, The same section was subsequently incubated with anti-CD68; macrophages are indicated by green fluorescence. **C**, The 2 stains were superimposed, resulting in a distinct yellow-orange staining (arrow). **D**, Colocalization at a higher magnification. (Original magnification  $\times 250$  in **A**, **B**, and **C**;  $\times 600$  in **D**.)

tissue samples from 5 patients with early inflammatory arthritis, including RA and other diagnostic categories. The level of mRNA expression was similar in the various arthropathies that were examined (Figure 5A). RNA,

extracted from 2 primary RA FLS cultures and from SMECs, was also evaluated by RT-PCR for FPRL1 mRNA expression (Figure 5A). RT-PCR demonstrated the presence of A-SAA in these cell types. The level of



**Figure 5.** Semiquantitative analysis of formyl peptide receptor-like 1 (FPRL1) mRNA expression in inflamed human synovial tissue from patients with various arthritides and in synovial membrane endothelial cells (SMECs). Total RNA was analyzed by reverse transcription-polymerase chain reaction using primers specific for acute-phase serum amyloid A and GAPDH. **A**, Representative example of FPRL1 mRNA expression in synovial tissue obtained from individual patients with various arthritides: psoriatic arthritis (PsA), rheumatoid arthritis (RA), undifferentiated arthritis (UA), and sarcoid arthritis (SA). Basepair (BP) markers are also shown. **B**, Representative examples demonstrating FPRL1 mRNA expression in primary RA-cultured fibroblast-like synoviocytes. **C**, Detection of FPRL1 mRNA in SMECs.

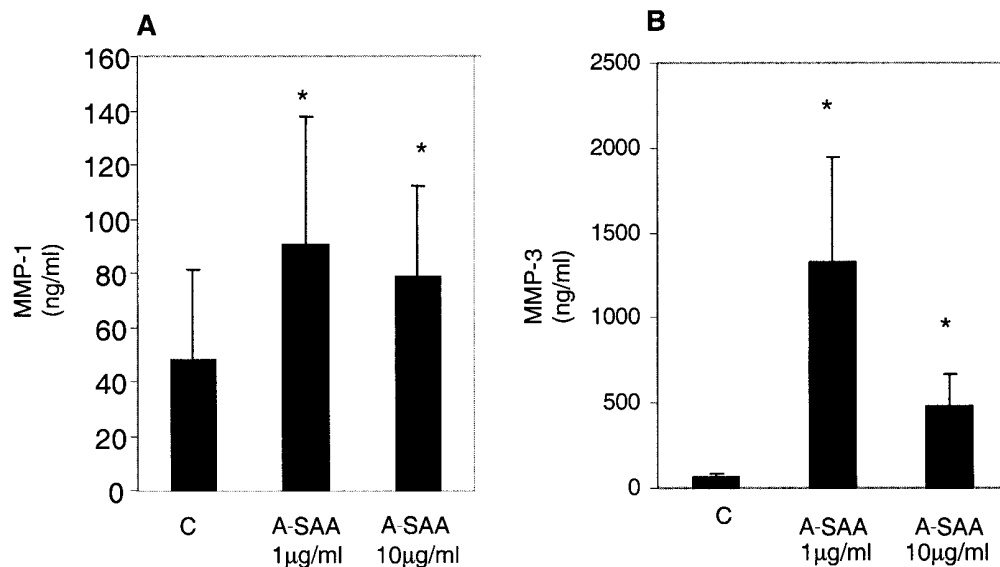


**Figure 6.** Regulation of acute-phase serum amyloid A (A-SAA) mRNA (A) and formyl peptide receptor-like 1 (FPRL1) mRNA (B) by proinflammatory mediators. Representative examples of polymerase chain reaction (PCR) products generated using primary fibroblast-like synoviocytes, which were maintained under normal conditions (C) or were stimulated over 24 hours with 10 ng/ml of tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), 10 ng/ml of interleukin-1 $\beta$  (IL-1 $\beta$ ), and 10 ng/ml of IL-6. PCR products generated were confirmed by Southern blot analysis using cDNA probes for human A-SAA and FPRL1.

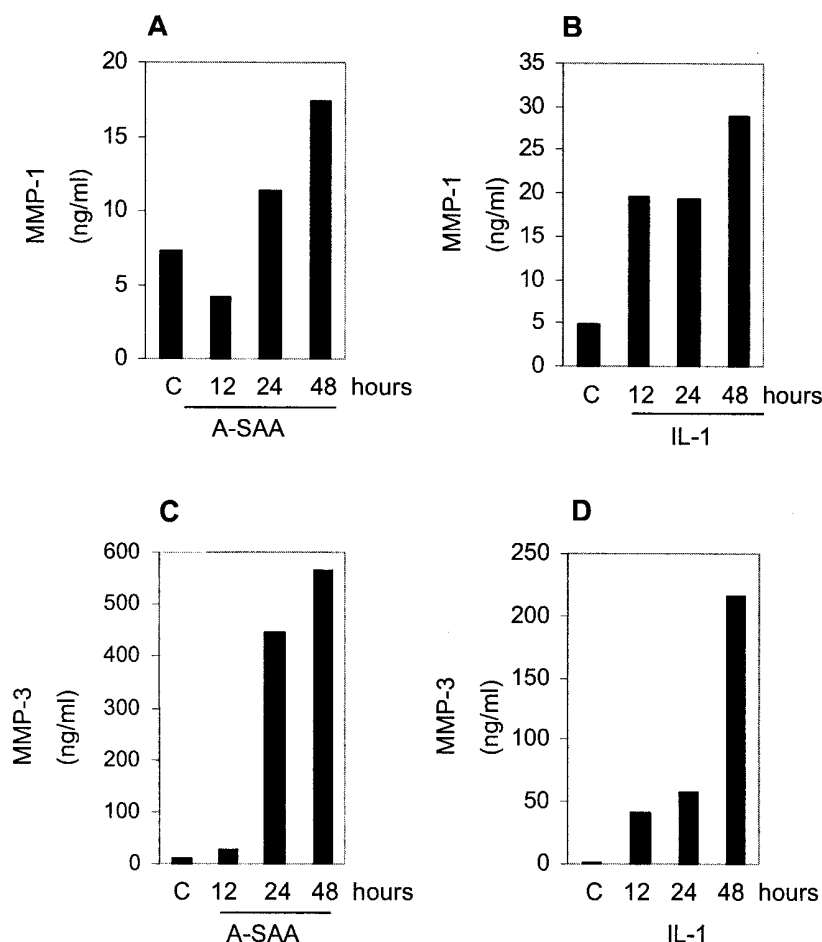
A-SAA mRNA detected in SMECs was low, possibly due to the low numbers of SMECs that were cultured (Figures 5B and C).

**Regulation of A-SAA gene expression by proinflammatory cytokines.** The importance of A-SAA and its receptor in inflammatory joint disease was further examined by investigating the ability of proinflammatory agonists to regulate A-SAA and FPRL1 mRNA levels in primary FLS. FLS were treated with TNF $\alpha$  (10 ng/ml), IL-1 $\beta$  (10 ng/ml), and IL-6 (10 ng/ml) for 4, 10, and 24 hours. RT-PCR analysis revealed that A-SAA mRNA expression was up-regulated in a time-dependent manner by TNF $\alpha$  and to a lesser extent by IL-1 $\beta$  (Figure 6A). In contrast, IL-6 had little effect on A-SAA mRNA levels in these FLS. RT-PCR analysis revealed that FPRL1 mRNA regulation is similar to that of A-SAA mRNA (Figure 6B). A-SAA mRNA and FPRL1 mRNA are coordinately up-regulated by TNF $\alpha$  and to a lesser extent by both IL-1 $\beta$  and IL-6. The levels of GAPDH mRNA remained constant under all of the conditions tested (Figure 6).

**The effects of A-SAA on metalloproteinase production.** In order to investigate the biologic effect that A-SAA protein has on MMP production, FLS were treated with A-SAA at various concentrations over time. MMP-1 and MMP-3 levels were measured in harvested



**Figure 7.** Dose-response of A-SAA induction of total matrix metalloproteinase 1 (MMP-1) (n = 5) (A) and total MMP-3 (n = 7) (B) in fibroblast-like synoviocytes (FLS) with a concentration range of recombinant SAA (A-SAA) protein. FLS were untreated (C) or were stimulated with A-SAA at either 1  $\mu$ g/ml or 10  $\mu$ g/ml. Total MMP-1 or MMP-3 concentrations in FLS supernatants were measured by enzyme-linked immunosorbent assay. Bars show the mean and SEM. \* =  $P < 0.05$ ; see Figure 6 for other definitions.



**Figure 8.** Total MMP-1 (A and B) and MMP-3 (C and D) protein secretion by FLS from a representative individual patient, following stimulation with either recombinant SAA (A-SAA) (10  $\mu$ g/ml) or interleukin-1 $\beta$  (IL-1 $\beta$ ; 10 ng/ml), respectively, over 48 hours. An enzyme-linked immunosorbent assay was used to determine the levels of total MMP-1 or MMP-3 protein secreted into the supernatants from FLS. See Figure 7 for other definitions.

supernatants following stimulation with A-SAA or IL-1 $\beta$ , a potent inducer of MMPs (27). Cultured FLS were treated with varying concentrations of A-SAA ranging from 0.1  $\mu$ g/ml to 150  $\mu$ g/ml. A-SAA at 1  $\mu$ g/ml and 10  $\mu$ g/ml consistently induced MMP-1 and MMP-3. Thus, these 2 concentrations of A-SAA were used in subsequent experiments, and the concentration used is indicated. Figure 7A illustrates significant ( $P < 0.05$ ) MMP-1 responses from 5 individual experiments comparing basal levels to the effects of stimulation with 1  $\mu$ g/ml and 10  $\mu$ g/ml of A-SAA. Figure 7B shows the MMP-3 responses from 7 individual experiments ( $P < 0.05$ ) comparing basal levels to induced levels following stimulation with 1  $\mu$ g/ml and 10  $\mu$ g/ml of A-SAA.

**Effect of A-SAA on metalloproteinase production over time.** In order to examine the time responses of MMP-1 and MMP-3 production, FLS ( $n = 5$ ) were treated with 10  $\mu$ g/ml of A-SAA or 10 ng/ml of IL-1 $\beta$  over a total of 60 hours. Figures 8A and B illustrate the effects of A-SAA and IL-1 $\beta$ , respectively, on MMP-1 protein production from representative FLS over 48 hours. Consistently, IL-1 $\beta$  had a greater effect on MMP-1 production than did A-SAA in all FLS ( $n = 5$ ). Figures 8C and D represent the effects of IL-1 $\beta$  and A-SAA, respectively, on MMP-3 protein production in individual FLS. Significant ( $P < 0.05$ ) changes in IL-1 $\beta$ -induced MMP-1 levels were observed by 12 hours, whereas A-SAA-induced MMP-3 production increased



**Table 1.** MMP-1 production following stimulation with A-SAA or IL-1 $\beta$ \*

Sample	MMP-1 production, ng/ml			Fold increase	
	Basal level	A-SAA, 1 $\mu$ g/ml	IL-1 $\beta$ , 10 ng/ml	A-SAA, 1 $\mu$ g/ml	IL-1 $\beta$ , 10 ng/ml
RA 1	7.3	11.3	51.2	1.5	6.9
RA 2	11.2	8.4	96.1	ND	8.5
RA 3	29.5	57.8	128.6	1.9	4.3
UA 1	5.2	14.8	35.1	2.8	6.7
UA 2	13.6	53.5	82.2	3.9	6.1
RA 1 explant	0.8	1.6	9.0	2	10.9
RA 2 explant	8.0	46.4	80.2	5.9	10.2

\* Following acute-phase serum amyloid A (A-SAA) stimulation, the mean  $\pm$  SEM fold increase in matrix metalloproteinase 1 (MMP-1) production was  $2.6 \pm 0.7$   $\mu$ g/ml ( $P = 0.042$  versus basal levels). For interleukin-1 $\beta$  (IL-1 $\beta$ ), the mean  $\pm$  SEM increase in MMP-1 production was  $7.6 \pm 0.9$  ng/ml ( $P = 0.018$  versus basal levels). RA = rheumatoid arthritis; ND = not determined; UA = undifferentiated arthritis.

at a later time point (24 hours) in all experiments ( $n = 5$ ). Both MMP-1 and MMP-3 production continued for up to 48 hours (Figure 8) and remained high at 60 hours (results not shown).

Table 1 shows data from 5 experiments that measured basal, IL-1 $\beta$ -induced, and A-SAA-induced MMP-1 production in FLS following stimulation with 10 ng/ml of IL-1 $\beta$  or 1  $\mu$ g/ml of A-SAA over 24 hours. MMP-1 production by synovial tissue explants from 2 patients undergoing arthroplasty was also examined. In summary, these experiments demonstrated that A-SAA induced a mean 2.6-fold increase in MMP-1 production over basal levels ( $P = 0.042$ ), compared with a mean 7.6-fold increase by IL-1 $\beta$  ( $P = 0.018$ ). Table 2 shows

**Table 2.** MMP-3 production following stimulation with A-SAA or IL-1 $\beta$ \*

Sample	MMP-3 production, ng/ml			Fold increase	
	Basal level	A-SAA, 1 $\mu$ g/ml	IL-1 $\beta$ , 10 ng/ml	A-SAA, 1 $\mu$ g/ml	IL-1 $\beta$ , 10 ng/ml
RA 1	0.4	2.6	76.7	6.3	191.3
RA 2	11.6	9.4	96.8	ND	8.3
RA 3	14.1	49.5	590.0	3.5	42.0
UA 1	55.8	1,756.4	2,271.5	31.5	40.7
UA 2	495.3	ND	703.1	ND	1.4
RA 1 explant	34.9	287.9	160.2	8.3	4.5
RA 2 explant	119.0	383.2	591.9	3.2	4.9

\* Following acute-phase serum amyloid A (A-SAA) stimulation, the mean  $\pm$  SEM fold increase in matrix metalloproteinase 1 (MMP-1) production was  $10.6 \pm 5.3$   $\mu$ g/ml ( $P = 0.056$  versus basal levels). For interleukin-1 $\beta$  (IL-1 $\beta$ ), the mean  $\pm$  SEM increase in MMP-1 production was  $41.9 \pm 25.8$  ( $P = 0.018$  versus basal levels). RA = rheumatoid arthritis; ND = not determined; UA = undifferentiated arthritis.

MMP-3 production in the same experiments following stimulation with 10 ng/ml of IL-1 $\beta$  or 1  $\mu$ g/ml of A-SAA for 24 hours. MMP-3 production by synovial tissue explants from 2 patients undergoing arthroplasty was also examined. These experiments demonstrated that A-SAA induced a mean 10.6-fold induction ( $P = 0.056$ ) and IL-1 $\beta$  induced a mean 41.9-fold induction ( $P = 0.018$ ). It is noticeable that there is wide interpatient variation between the basal and induced levels of MMP-1 and MMP-3 production. However, the basal, IL-1-induced, and A-SAA-induced levels of MMP-3 were consistently higher than those observed for MMP-1.

## DISCUSSION

This study confirms and extends results of previous studies, which demonstrated A-SAA gene expression and protein production by FLS isolated from the joints of patients with RA (12,13). First, A-SAA mRNA expression was demonstrated in FLS from patients with other inflammatory arthropathies, including PsA, undifferentiated arthritis, and sarcoidosis. Moreover, evidence is provided to confirm that A-SAA is produced not only by FLS, but also by synovial tissue macrophages and endothelial cells. Second, the demonstration of FPRL1, an A-SAA receptor, in FLS from inflamed joints, and its up-regulation by TNF $\alpha$ , suggests that A-SAA may contribute locally to the altered molecular and cellular physiology in inflammatory joint diseases. Finally, it was demonstrated that A-SAA can induce both MMP-1 and, more strikingly, MMP-3 protein production by human FLS.

Hepatocytes are a major source of A-SAA synthesis (28). However, extrahepatic production of A-SAA has been demonstrated in many normal human tissues, including breast, the gastrointestinal tract, lung, skin, prostate, thyroid, tonsil, placenta, and brain (9). The demonstration of extrahepatic A-SAA mRNA expression by FLS and synovial tissue endothelial cells was an important component of this study and is consistent with previous studies of A-SAA mRNA expression by endothelial and smooth muscle cells in human atherosclerotic lesions (11). Using immunohistochemical techniques, A-SAA protein production by synovial tissue macrophages was also demonstrated, and this is consistent with studies that demonstrated abundant A-SAA production by macrophage/monocyte cell lines (29). Attempts to isolate synovial tissue macrophages for molecular studies were unsuccessful. In-situ hybridization techniques might have confirmed the presence of A-SAA mRNA in

individual cell populations but were not undertaken in this study.

A second important finding in this study was the coordinate up-regulation of both A-SAA and FPRL1 mRNA by proinflammatory cytokines in cell populations isolated from inflamed synovial tissues. Because FPRL1 is a known A-SAA receptor (14), this observation suggests important functional roles for A-SAA as a ligand, and for FPRL1 as a receptor, in inflammatory arthritis. In previous studies, FPRL1 mRNA expression was demonstrated mainly on monocytes and neutrophils (30,31). FPRL1 is also expressed on nonphagocytic cells, including hepatocytes and epithelial cells (15). Furthermore, A-SAA binding sites have been demonstrated on human peripheral blood CD3+ T lymphocytes, but it is not clear whether these binding sites represent FPRL1 or additional, unidentified A-SAA receptors. A-SAA, in concentrations corresponding to those found during acute inflammatory episodes, is known to induce proinflammatory activities, including integrin expression and activation, cell adhesion, chemotaxis, phagocytosis, and tissue infiltration of monocytes, neutrophils, lymphocytes, and mast cells (30,32,33), which can be mediated through FPRL1 (14).

The lipid metabolite lipoxin A<sub>4</sub> also binds to FPRL1 but transduces inhibitory signals (34,35), which antagonize the proinflammatory responses induced by agonist FPRL1-binding peptides and by proinflammatory mediators such as TNF $\alpha$  (36). The use of FPRL1 by A-SAA to chemoattract leukocytes may act to recruit these cells to participate in the degradation of A-SAA. The use of FPRL1 by amyloidogenic protein and peptide agonists suggests that this receptor plays a crucial role in the proinflammatory aspects of systemic amyloidosis and neurodegenerative and inflammatory joint disease (37).

The final aim of this study was to investigate A-SAA induction of MMPs in the setting of inflammation. A-SAA was shown to stimulate MMP-1 and MMP-3 production by FLS. In previous studies, A-SAA-induced MMP-1 production by normal rabbit FLS (20), and A-SAA-induced MMP-2 and MMP-3 production by FLS from patients with RA, were demonstrated (18). In this study, total MMP-1 and MMP-3 concentrations were quantified, so that the pro- and active MMP levels, as well as the complexed inhibitor/MMP levels, were included in the final values. Measurement of the total MMP levels best reflected the rate of MMP synthesis. In the experimental conditions employed, A-SAA increased MMP-1 production by a mean 2.5-fold and increased MMP-3 production by ~9-fold.

Not surprisingly, there was considerable patient-to-patient variation in constitutive and inducible MMP production. The dose-response curve observed was characteristic of ligands that use G protein-coupled receptors, with suboptimal responses at higher doses (38). This is thought to be attributable to the mechanism whereby G protein-coupled receptors adapt to long-term stimuli by reducing their response. IL-1 $\beta$ -induced MMP-1 and MMP-3 values were ~3-fold and 5-fold greater than A-SAA-induced values, respectively. In previous studies, IL-1 $\beta$  was shown to be a potent mediator of MMP production (39,40).

In this study, IL-1 $\beta$ -induced MMP-1 and MMP-3 production was consistently observed after 12 hours, whereas A-SAA-induced MMP production was not observed until 24 hours after stimulation. The greater magnitude of the response to IL-1 $\beta$ , and the delayed response to A-SAA, suggested the possibility that A-SAA-induced MMP production might be mediated through IL-1 signaling pathways. The possibility that A-SAA induction of MMP-1 and MMP-3 might occur through different pathophysiologic pathways is consistent with the previous demonstration of maximal A-SAA-induced MMP-1 production by IL-1 $\beta$ -activated rabbit fibroblasts (20), and by the failure of anti-IL-1 $\beta$  antibodies to block A-SAA-induced MMP-3 production by RA FLS (18). In inflamed synovial tissue exposed to high concentrations of IL-1 $\beta$ , FLS are likely to be in a high state of activation, facilitating A-SAA-induced MMP-1 production.

Compared with other measures of the acute-phase reaction, serum levels of A-SAA best differentiated RA from other categories of inflammatory arthritis (41). In addition, changes in serum A-SAA levels best reflected the changes in disease status. These observations are explained by the abundant synovial tissue (as well as hepatic) production of A-SAA in RA, which usually is characterized by many actively inflamed joints. PsA, undifferentiated arthritis, and other inflammatory arthropathies usually manifest with significantly fewer inflamed joints. MMP-1 plays a critical role in the pathophysiology of joint erosion in human and experimental arthritis (24,42–44). In this study, A-SAA induction of MMP-1 was modest compared with that of MMP-3, suggesting that A-SAA expression and synthesis in synovial tissue may have a limited direct effect on MMP-1-mediated mechanisms of joint damage. The preferential induction of MMP-3 by A-SAA is consistent with the associations that have been reported in clinical studies of RA between MMP-3 and measures of inflammation, but not measures of joint erosion (42,45). Thus,

the role of MMP-3 in joint damage remains unclear. In experimental arthritis, inactivation of the MMP-3 gene had no effect on cartilage loss or proteoglycan staining (46). The role of MMP-3 in synovial tissue inflammation also remains unclear, but it may be related to the other inflammatory events, modulating adhesion molecule expression, chemotaxis of monocytes and neutrophils, and phagocytosis, that have been associated with the expression and synthesis of A-SAA and FPRL1. In conclusion, the observations in this study suggest that therapeutic targeting of A-SAA or FPRL1 may modulate some of the critical pathophysiologic pathways that are involved in progressive inflammatory diseases.

### ACKNOWLEDGMENTS

We thank the late Dr. Leanne Stafford, as well as Drs. Eithne Murphy and David Kane for their assistance in acquiring the synovial tissue samples used in this study. We also thank Nicola Cassidy and Martina Gogarty for their technological excellence in preparing the tissues for analysis.

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